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Stomatal Frequency, Distribution, and Needle Hydrophobicity in Cloud Forest Spruce and Fir, Southern Appalachian Mountains

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Cover Page Footnote

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I. Introduction

Stomata are pore-like structures located on the leaf surfaces of virtually all vascular, terrestrial plant leaves, and are responsible for the uptake of photosynthetic CO_2, as well as for the potentially detrimental water loss (transpiration) from inside the leaf (MacDonald 2002). Thus, stomata play a primary role in regulating carbon uptake for growth and the prevention of plant desiccation (Apple et al. 2000; Croxdale 2000). Moreover, the frequency of these structures on leaf surfaces can dictate the degree of gas exchange potential for both photosynthetic CO_2 uptake and transpirational water loss. In response to favorable environmental signals, stomata may open to facilitate carbon uptake, or close to prevent tissue drying and the maintenance of higher water use efficiency at different times of the growth season or given time of day (Croxdale 2000). Physical obstruction of these structures by a water film can strongly inhibit gas exchange (Brewer et al. 1991; Brewer and Smith 1997) and significantly decrease photosynthetic carbon gain by the plant (Brewer and Smith 1995). Water film coverage of stomata could be ever-present issue in the mountaintop spruce-fir forests of the Appalachian Mountains in the eastern United States, a region characterized by frequent cloud immersion and high humidity that would enhance water condensation on leaf surfaces.

No studies to our knowledge have investigated the stomatal frequency on the leaf surface and hydrophobicity of leaves in the dominant conifer tree species *P. rubrens* and *A. fraseri*, and, thus, the potentially strong impacts on photosynthetic gas exchange and growth due to frequent cloud immersion. In the current study, we hypothesized that the recognized difference in altitudinal distribution of the dominant fir [Fraser fir, *Abies fraseri* (Pursh) Poir] and spruce (red spruce, *Picea rubens* Sarg.) trees corresponded to a difference in stomatal frequency and surface hydrophobicity between the two species and elevations. Fir is found in greater numbers at higher higher elevations where cloud-immersion is also more frequent (Braun 194). To test this hypotheses, the stomatal frequency (number per unit area) and surface hydrophobicity were measured in the spruce and fir at high and low elevation sites.

2. Materials and Methods

The general methodology consisted of comparing the two dominant conifer tree species at a higher elevation site with a lower site that experiences substantially fewer cloud-immersed days during a year (Johnson and Smith 2008, Reinhart and Smith 2008). Leaves (needles) were examined for stomatal frequency (number per unit area) and arrangement, plus surface wettability.

Study sites

Two study sites were selected near Mt. Mitchell, the highest peak of the southern Appalachian Mountains, USA. A high elevation site at 2044 m elevation was selected (35°5'51" N, 82°15'50" W) along with a low elevation site at 1460 m (35°42'54" N, 82°16'23" W) in Pisgah National Forest. Two needles from five trees of each of the two species were selected at the high and low elevation sites for stomatal peels (impressions) and microscopy evaluation of stomatal frequency and needle surface morphology. Two micrographs of each needle were also taken that displayed the full length of the needle so that any variation in stomatal frequency along the length of the needle could be identified (Hultine and Marshall 2000; Apple et al. 2000). Leaf surface impressions of each needle were generated using impressions (peels) created by a dried coating of nail polish (Brewer and Smith 1997; Neill, Neill and Frye 1990) and examined using a Zeiss Axioplan Upright microscope with a 40× objective. Stomatal frequency (number per unit area) was determined using Image J analysis.

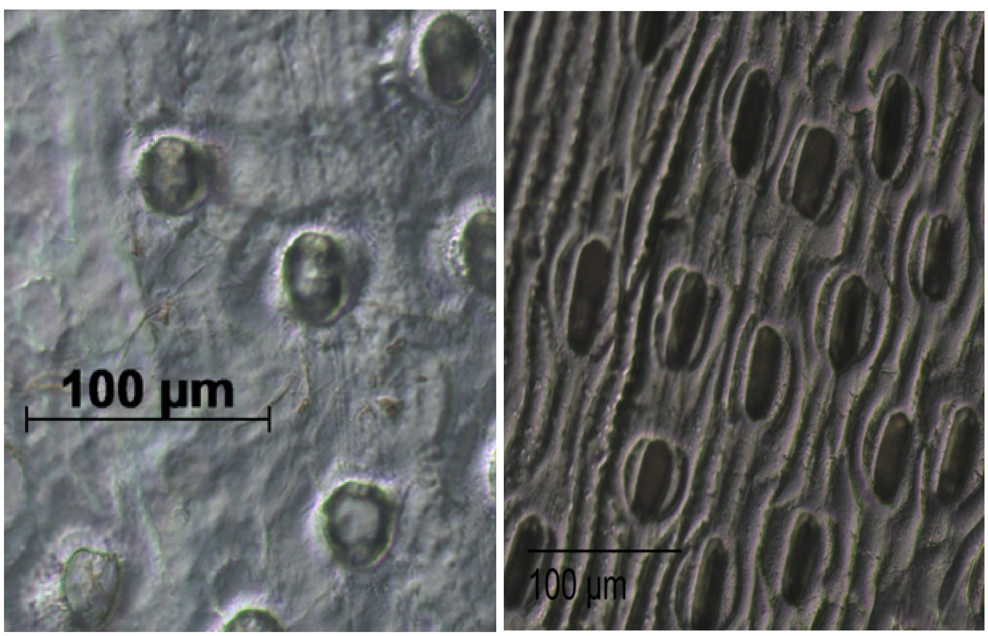
Twenty spruce needles were sampled at each elevational site from 10 different individuals to examine leaf surface hydrophobicity (water repellency), along with forty fir needles from each elevation to yield 20 mean values for both adaxial (top) and abaxial (bottom) leaf surfaces. A Tantec contact angle meter was used to measure the contact angle of 2 μl droplets of distilled water (placed by pipette) on the surface of sampled needles. The contact angle (θ) is the measure of the angle between the needle surface and line tangent to the droplet where the droplet meets the surface (Brewer et al. 1991; Brewer and Smith 1997). This measurement of θ is a quantitative measure of the degree of hydrophobicity (degree of water repulsion) whereby a greater θ indicates a more hydrophobic surface (ChemInstruments 2011).

Statistics

Microsoft Excel was used to compute a series of Student's t tests ($P \leq 0.05$) to determine the statistical significance (*p* values*)* between the differences in all mean values among individual leaf measurements between and within a species for both stomatal frequency and θ, and between the two elevations

3. Results

The observed pattern in stomatal distribution in *P. rubrens* (red spruce) was three linear, evenly spaced rows that ran the length of the needle (Figure 1). These stomatal rows were also located in trench-like depressions on the needle surface. *Abies fraseri* stomata occurred on the abaxial surface in two linear rows that ran the length of the needle. Stomata were also observed on adaxial surfaces in a thin row near the needle tip.



P. rubra *A. fraseri*

Figure 1. Photomicrographs of stomatal size and distribution pattern on the epidermis of the spruce (*P. rubens*) and fir (*A. fraseri*) species in this study.

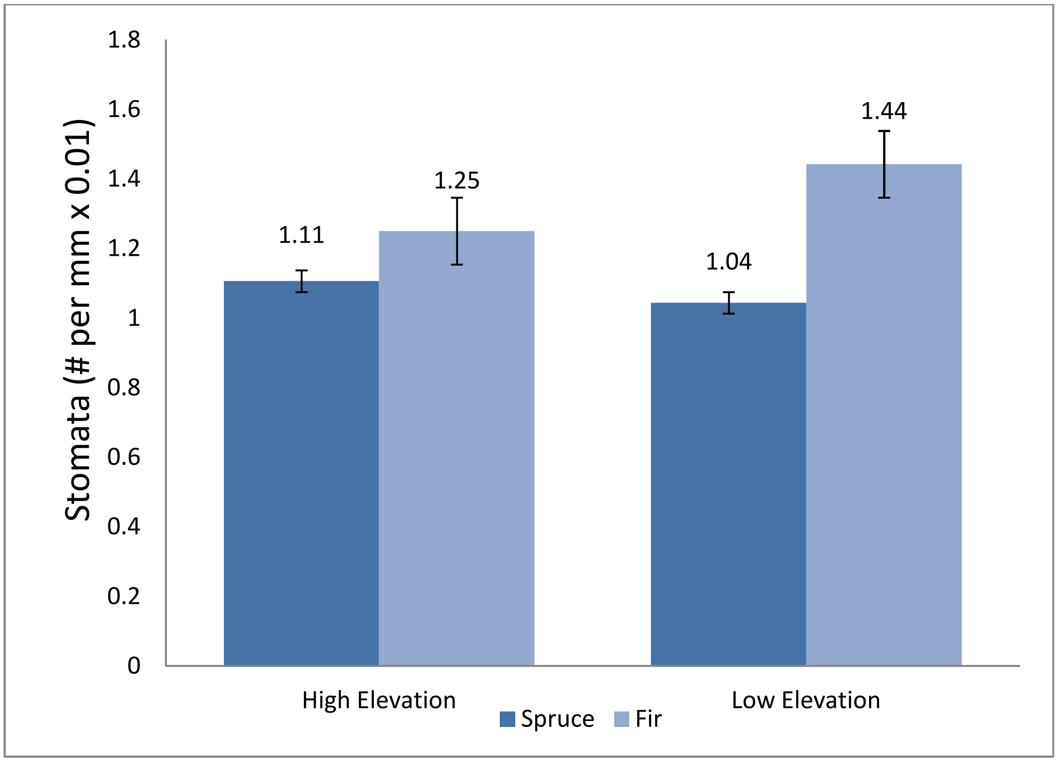


Figure 2. Mean stomatal frequencies (# per mm^2) on spruce and fir needles at high and low elevations. No significant difference between elevations was observed and no differences between species at high elevation. Stomatal number per unit leaf area in spruce needles were observed to be significantly less than those in fir needles at low elevation .

No statistically significant differences in stomatal frequency occurred between high and low elevations in either spruce or fir needles (p = 0.096, 0.322, respectively); no statistically significant differences in stomatal frequency were observed between fir and spruce needles at the higher elevation site (p = 0.0813), while stomatal frequencies in spruce needles were less than those for fir needles at the lower elevation site (p = 0.001) (Figure 2).

No statistically significant differences in mean droplet contact angle, θ (leaf hydrophobicity) were observed between the higher and lower elevation sites (p = 0.316414, 0.461 and 0.179, respectively), or between the two species (p = 0.819 and 0.789, respectively). Abaxial surfaces of fir needles had contact angles similar to those of spruce, while adaxial leaf surfaces had smaller θ (less hydrophobic) than those of spruce (p = 0.00075 and 0.0319, respectively).Abaxial sides of fir needles had greater θ (more hydrophobic) than those of adaxial surfaces ($p = 0.0001$ and 0.0371, respectively) (Figure 3).

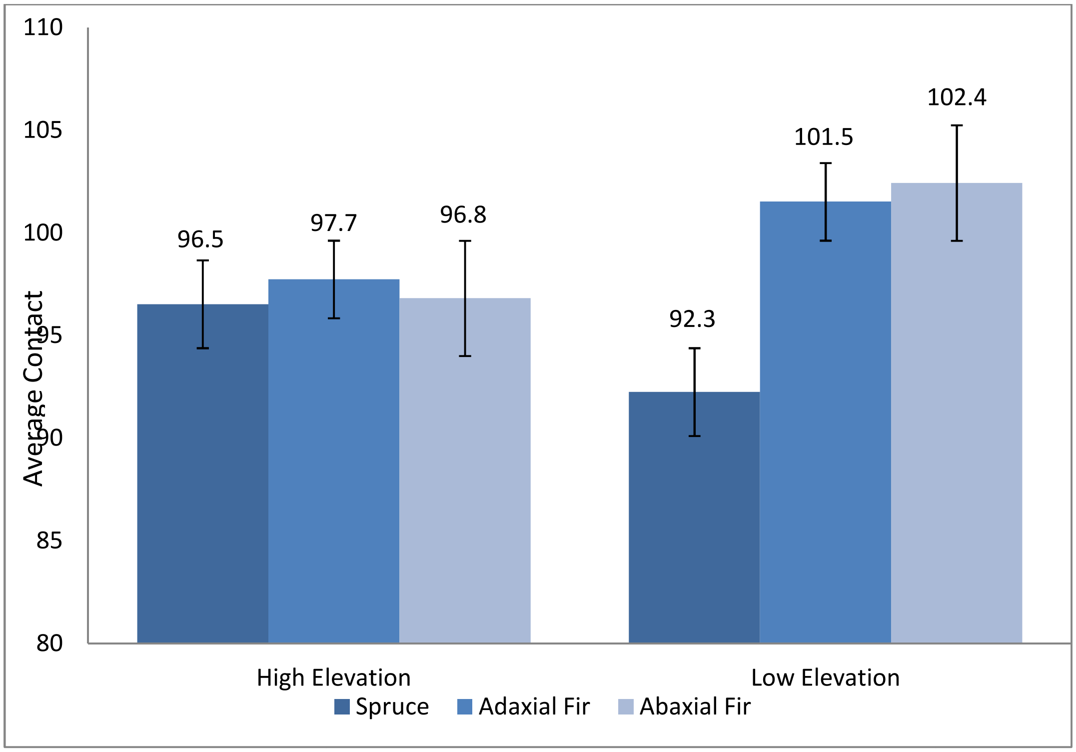


Figure 3. Mean contact angles θ of spruce and fir needle surfaces at high and low elevations. No statistically significant differences in contact angle were observed between high and low elevations. Abaxial surfaces of fir needles had contact angles similar to those of spruce ($P = 0.82$, *0.79*), while adaxial surfaces of fir needles had θ smaller than those of spruce needles . Abaxial fir needles had significantly greater contact angles than those of adaxial surfaces of fir needles.

4. Discussion

A variety of epidermal structures such as epicuticular wax, trichomes (leaf hairs) and textured surfaces have been shown to effectively repel water in broad-leaved plants by the formation of small water droplets, yet, can also retain these droplets on the leaf surface (Brewer and Smith 1995, Brewer and Smith 1997, Brewer et al. 2001). As a result, both the prevention of water films and the retention of the droplets on the leaf surface act to sustain CO_2 uptake for photosynthesis while saturating the air boundary layer next to the leaf with water vapor. This results in a major reduction in transpiration and increase in water use efficiency In addition, previous studies have reported decreases in stomatal frequency with increasing altitude (e.g. Hultine and Marshall 2000), suggesting that conifers with lower stomatal frequencies may offset the drier atmosphere and greater mass diffusion rates at higher elevations (Smith and Geller 1979). This is supported by numerous other reports of environmental effects on intraspecific stomatal frequency (e.g. MacDonald 2002). Finally, at our research sites in the southern Appalachian Mountains, and elsewhere, a greater abundance of fir occurs at higher elevations compared to the spruce (Sullivan and Pimillo 1988).

Stomatal Pattern

The stomatal patterns observed in this study are consistent with literature documenting the relatively large variety found in gymnosperms (Stockey and Taylor 1978, Croxdale 2000). These studies have also suggested that these stomatal patterns allow CO_2 uptake in areas near internal mesophyll cells that perform photosynthesis (Croxdale 2000), possibly explaining the non-random distribution of stomata. The fact that stomata were observed exclusively within linear rows on needles in both species is similar to past investigations showing patchy stomatal patterns instead of homogenous configurations (Beyschlag et al. 1993). The fact that the fir and spruce species observed here had distinct stomatal patterns on the leaf surface suggests that the interior configurations of photosynthetic cells involved in gas exchange are also different. This relationship between stomatal pattern and connection to internal, photosynthesizing cells has not been investigated, to our knowledge, in any conifer species.

Stomatal Frequency

Our findings that stomatal frequency did not change for either species at the higher and lower elevation sites is contradicted by literature reporting a decrease in stomatal frequency at higher elevations in four dominant conifer tree species of the central Rocky Mountains, USA (Hultine and Marshall 2000). It is possible that the two field sites studied here were not different enough in altitude to cause differences in stomatal frequencies or θ.

Stomatal frequencies between the spruce and fir needles were statistically comparable in samples collected at the high elevation site, but significantly different at the low elevation site. This finding is supported by past observations whereby variable stomatal densities were attributed to variability in micro-environmental conditions (MacDonald 2002). One such condition is the concentration of carbon dioxide in the atmosphere (MacDonald 2002; Kouwenberg et al. 2003), an environmental characteristic which decreases with greater elevation, along with corresponding decreases in stomatal frequency in conifer needles (Apple et al. 2000; Hultine and Marshall 2000; Kouwenberg et al. 2003).

Surface Hydrophobicity

The data presented here suggest that the spruce and fir needle surfaces had a comparable level of hydrophobicity, although the adaxial fir surfaces are slightly more hydrophobic (greater θ). This idea is consistent with reports that the leaf surfaces with greater stomatal frequency are also more efficient at repelling water (Smith and McLean 1989). Because stomata facilitate photosynthetic gas exchange, it follows that leaf surfaces displaying larger stomatal densities would display a greater hydrophobicity than those with fewer stomata per surface area, as observed in this experiment. Levels of photosynthetic gas exchange were observed to vary depending on the length of time during which the water coverage was present, as well as on the frequency of exposure to moisture coverage (Brewer and Smith 1997).

The fact that spruce needles are cylindrical, instead of planar, causes water droplets to run off the leaf upon contact with the needle surface. Water is not retained on the surface of spruce needles, as it is on the adaxial surfaces of fir leaves, because stomata are oriented on every plane of the spruce needle surface, suggesting that if spruce needles were to retain water by a mechanism similar to that of fir needles, stomata would be covered by water, thus inhibiting gas exchange. The orientation of needles on the branch could also affect the retention and channeling of water.

Adaxial Stomata

Previous experiments suggest that needle surfaces facilitate the uptake of water in environments that routinely experience fog and precipitation (Limm et al. 2009). It was proposed that water uptake was facilitated by leaf tissue, rather than by the roots, because the frequent cloud immersion most successfully covers the plants' leaves without reaching the roots. While past results provide justification for the abundance of stomata on the abaxial versus adaxial surfaces of fir needles, they present little explanation regarding the presence of stomata on the adaxial tip. Based on the premise that stomatal frequency is greatest on the abaxial (lower) surfaces to better avoid rain and dewfall, it is reasonable to expect an absence of stomata on the adaxial (upper) surface. It is possible that stomata located on the adaxial surfaces serve a purpose different to that of those located on the abaxial surfaces (Limm et al. 2009)..

Altitudinal Variation

The results of the current investigation do not support the hypothesis that each species distribution along the elevation gradient may be influenced by stomatal frequencies and/or θ. Because the respective hydrophobicities of abaxial fir needle surfaces and spruce needle surfaces were statistically similar, despite their differences in abundance, these data suggest either of the following: a) the difference in elevational distribution is not a reflection of the plant's hydrophobic response to increased cloud immersion at higher elevation , or b) the cloud immersion conditions of both the lower and higher field sites are similar. This idea is supported by previous research in which hydrophobicity and water repellence within one species differed between open and sheltered habitats (Brewer and Smith 1997). Species in open habitats where dew fall was greater were observed to display greater values of θ, plus greater stomatal and trichome densities, indicating that environmental factors may influence leaf water repulsion. Another consideration of the results presented here is that the non-significant data comparisons may also be the result of a lack of genetic differentiation within these two proximal populations, as opposed to the study of Hultine and Marshall (2000) where their populations were separated by hundreds of kilometers.

Epicuticular Wax

The distribution and quantity of epicuticular wax vary according to species, individual plants, and even regions of the same plant (Hanover and Reicosky 1971). Evidence suggests that two kinds of epicuticular wax exist on conifer needles, defined in the literature as structural and amorphous waxes (e.g. Hanover and Reicosky 1971). Stomata have also been observed to be covered by varying degrees by surface wax and sometimes filled with pencil-like wax plugs (Field et al. 1998; Koch et al. 2008). It has been suggested that this wax serves as a barrier to gas exchange, especially during winter for evergreen tissue when frozen soils and stems prevent replacement of water evaporated through leaf cuticles (Hanover and Reicosky 1971). In an experiment during which epicuticular wax deposits were chemically removed from the needle surface, stomata were reportedly situated in sunken, wax-filled depressions in the tissue surface (Stockey and Taylor 1978), another characteristic that could potentially serve to prevent stomata from being inundated by water during rain or dewfall. This investigation also documented variability in the degree to which stomata were sunken (Stockey and Taylor 1978). It is reasonable to predict that this variability is related to water exposure of specific areas on the needle, suggesting that stomatal depressions are yet another mechanism by which these conifers may avoid the inhibition of gas exchange by water film coverage.

Climatic Factors

Literature suggests that leaf morphology, including stomatal frequency (Croxdale 2000) and the formation of epicuticular wax, is affected by changes in atmospheric temperature and levels of carbon dioxide (Apple et al. 2000). Past investigations have observed a decrease in stomatal frequency in conifers grown under current atmospheric levels of carbon dioxide with respect to that of conifers grown under preindustrial conditions (Apple et al. 2000; Croxdale 2000). These findings are challenged by other investigations which observed few effects of climate change on stomatal frequency values, suggesting that conifers have a limited capacity to adapt to changing climate conditions due to a greater influence of genetic information than environmental factors (Apple et al. 2000; Croxdale 2000).

Recent increases in global air temperatures suggest a relative increase in atmospheric humidity, a change that could also result in higher levels of cloud immersion. This climatic change would likely manifest itself as a relative increase in condensation on conifer needles, making the effects of needle surface anatomy and hydrophobicity even more pertinent. In addition, these data might be applied to increasing the growth and water use efficiency of agricultural systems that employ spray irrigation techniques to nurture crops (Smith and McClean 1989), or to prevent the invasion of pathogens in numerous plant species (Brewer and Smith 1997).

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